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A novel, point-of-care, whole-blood assay utilizing dielectric spectroscopy is sensitive to coagulation factor replacement therapy in haemophilia A patients

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Abstract

Background: Reliable monitoring of coagulation factor replacement therapy in patients with severe haemophilia, especially those with inhibitors, is an unmet clinical need. While useful, global assays, eg thromboelastography (TEG), rotational thromboelastometry (ROTEM) and thrombin generation assay (TGA), are cumbersome to use and not widely available.

Objective: To assess the utility of a novel, point-of-care, dielectric microsensor – ClotChip – to monitor coagulation factor replacement therapy in patients with haemophilia A, with and without inhibitors.

Methods: The ClotChip T_{peak} parameter was assessed using whole-blood samples from children with severe haemophilia A, with (n = 6) and without (n = 12) inhibitors, collected pre- and postcoagulation factor replacement therapy. ROTEM, TGA and chromogenic FVIII assays were also performed. Healthy children (n = 50) served as controls.

Results: ClotChip T_{peak} values exhibited a significant decrease for samples collected postcoagulation factor replacement therapy as compared to baseline (pretherapy) samples in patients with and without inhibitors. A difference in T_{peak} values was also noted at baseline among severe haemophilia A patients with inhibitors as compared to those without inhibitors. ClotChip T_{peak} parameter exhibited a very strong correlation with clotting time (CT) of ROTEM, endogenous thrombin potential (ETP) and peak thrombin of TGA, and FVIII clotting activity.

Conclusions: ClotChip is sensitive to coagulation factor replacement therapy in patients with severe haemophilia A, with and without inhibitors. ClotChip T_{peak} values correlate very well with ROTEM, TGA and FVIII assays, opening up possibilities for its use in personalized coagulation factor replacement therapy in haemophilia.

KEYWORDS
blood coagulation tests, factor VIII, hemophilia A, point-of-care testing

1 | INTRODUCTION

Regular coagulation factor replacement therapy, delivered prophylactically, has revolutionized care for haemophilia.[1] As available therapies have become safer over the last few decades, management of haemophilia has moved from monitoring for infectious agents to monitoring factor levels in order to achieve target ranges necessary to avoid any bleeding events.[2] Recent advances in extension of half-life for factor VIII (FVIII) and factor IX (FIX) have made it possible to redefine our management goals, but have also forced clinicians to depend on individual patient pharmacokinetics to achieve these goals.[3] Laboratory assessment has always played an essential role, not only in diagnosis but also for monitoring adequacy of coagulation factor replacement therapy in haemophilia. Currently available factor assays, however, are unable to measure global clotting function, perhaps explaining why factor levels serve as poor predictors of an individual patient's bleeding risk or clinical phenotype.[4,5] Furthermore, for haemophilia patients with inhibitors, monitoring bypassing agent therapy is uniquely challenging. Global haemostasis assays, eg thrombin generation assay (TGA), thromboelastography (TEG), rotational thromboelastometry (ROTEM) and clot waveform analysis, can measure the missing function rather than the missing factor.[6] However, these global assays not only require expert interpretation, but also are technically challenging to operate, often unavailable at bedside and not widely available at all treatment centres.

We have recently reported on the development of ClotChip, a novel dielectric microsensor for rapid and comprehensive assessment of whole-blood coagulation based on the electrical technique of dielectric spectroscopy.[7,8] The ClotChip read-out has been shown to be sensitive towards detecting non-cellular (ie coagulation factor) and cellular (ie platelet) abnormalities in the haemostatic process, thereby allowing global haemostatic analysis in a miniaturized, portable measurement platform using miniscule blood sample volumes (<10 μL).[8]

We evaluated the performance of ClotChip in 15 healthy volunteers and 12 patients suffering from coagulation defects.[7] The ClotChip read-out exhibited superior sensitivity at distinguishing among coagulation disorders as compared to conventional screening coagulation tests.[7] Building on this, in the present study we assessed the utility of ClotChip in monitoring replacement therapy with coagulation factor concentrates in children with severe haemophilia A with and without inhibitors, and evaluated the correlation of the ClotChip read-out with FVIII clotting activity and extant global haemostasis assays such as the ROTEM and TGA.

2 | MATERIALS AND METHODS

2.1 | Study population

Following written informed consent/assent, children and young adults with haemophilia A with and without inhibitors were enrolled between January 2017 and July 2018 in a prospective study approved by the Institutional Review Board (IRB) of University Hospitals Cleveland Medical Center. Inclusion criteria included (a) birth to 21 years (included) with a diagnosis of severe haemophilia A (FVIII:C of <1%) with and without inhibitors, (b) non-bleeding state and (c) needing a dose of a standard or extended half-life FVIII, FEIBA or recombinant FVIIa at the time of consent. Exclusion criteria included an active bleeding state, diagnosis of more than one bleeding disorder and a need for more than one coagulation product. Subjects who met the inclusion criteria were administered an infusion of their clotting factor concentrate or a bypassing agent in the clinic or the emergency room. Normal, healthy children who were without an acute illness or chronic medical condition and had no personal or family history of bleeding or thrombotic disorders were also enrolled from a well-child ambulatory setting or sedation suite, as a control group for obtaining reference-range values. Final patient population accrued for the studies included children with severe haemophilia A (n = 12), children with haemophilia and inhibitors (n = 6), and normal, healthy children without coagulopathy (n = 50).

2.2 | Preparation of samples

Blood samples were collected by venipuncture using a 21-gauge needle, or from a central venous catheter (utilizing institutional protocol for waste), in 1.8-mL vacutainer tubes containing 3.2% sodium citrate anticoagulant (BD). Samples from patients with haemophilia were collected both prior to the initiation of factor replacement therapy and 15 minutes after administration of recombinant coagulation FVIII (40-60 IU/kg) for severe haemophilia A or a bypassing agent (FEIBA (100 U/kg) or recombinant FVIIa (90 μg/kg)) for haemophilia with inhibitors. For chromogenic FVIII activity and TGA measurements, whole blood was centrifuged twice in succession (2500 *g* for 15 minutes at room temperature) within an hour of collection to obtain platelet-poor plasma (PPP),[9] which was then stored at −80°C.

2.3 | ClotChip measurements

The ClotChip operation was based on measuring the dielectric properties of whole blood during the coagulation process in a disposable microfluidic sensor. The ClotChip featured a three-dimensional (3D) capacitive sensing area embedded in a microfluidic channel to extract the dielectric permittivity of whole blood. With a blood sample in the sensing area, the impedance of the sensor would change based on the dielectric permittivity of the sample. The ClotChip fabrication was based on a low-cost, batch-fabrication method of screen-printing gold electrodes on polymethyl methacrylate (PMMA) plastic substrates. Detailed descriptions of the ClotChip operation and fabrication steps have been previously reported in Ref.[8] Figure 1A shows a photograph of the fabricated ClotChip with dimensions of 26 mm × 9 mm × 3 mm and a total sample volume of 9 μL.

All ClotChip measurements were performed within 2 hours of blood collection. Three hundred microliters of citrated blood sample was warmed in a thermostatic chamber at 37°C for 15 minutes,

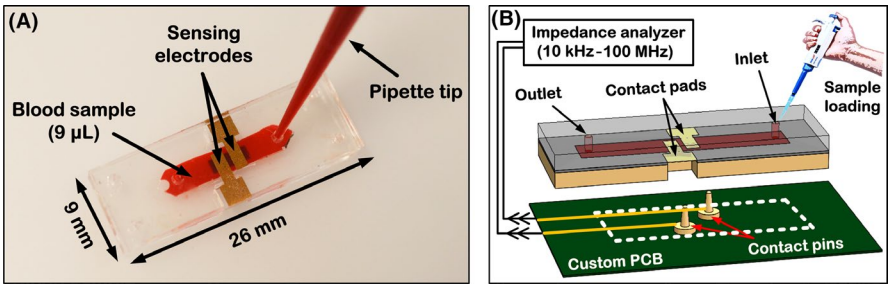

FIGURE 1 ClotChip design and experimental setup. A, Photograph of ClotChip sensor with sample volume of 9 µL. B, Schematic of experimental set-up demonstrating the use of spring-loaded contact pins to make electrical connections between the ClotChip and an impedance analyzer (Agilent 4294A)

followed by re-calcification with 25.6 µL of aqueous 250 mmol/L $CaCl_2$ (final Ca^{2+} concentration = 20 mmol/L). Thereafter, 10 µL of the sample was immediately injected into the sensor using a micropipette. Figure 1B shows a schematic illustration of the experimental set-up. Dielectric measurements were performed over a frequency range of 10 kHz-100 MHz at 10-second intervals inside a thermostatic chamber at 37°C. As described previously,[7,8] the dielectric properties of whole blood varied during the coagulation process, and a measurement frequency of 1 MHz was chosen to maximize the sensitivity to the coagulation process. Therefore, the ClotChip read-out was taken as the temporal variation in the real part of blood dielectric permittivity at 1 MHz. The ClotChip read-out characteristically rose to a permittivity peak, and the time to reach this peak (T_{peak}) was taken to be indicative of the coagulation time.[7,8]

2.4 | ROTEM measurements

Whole-blood coagulation profiles were concurrently recorded on a quad-channel computerized ROTEM Coagulation Analyzer (TEM International) using the non-activated TEM (NATEM) assay and following the manufacturer's directions. The ROTEM clotting time (CT) parameter – a clinically important indicator of a patient's coagulation status – indicated the initial detection of clot formation. In previous work, we have established a very strong positive correlation (r = 0.99, P < 0.0001, n = 9) between the ROTEM CT and ClotChip T_{peak} parameters using ex vivo-modified whole-blood samples from healthy volunteers.[8]

2.5 | Thrombin generation measurements

Thrombin generation assays were performed on PPP using Technothrombin TGA kit (Technoclone) with Reagent C (RC) Low and were read on SpectraMax M5 fluorescent plate reader (Molecular Devices). The approximate concentrations of recombinant tissue factor (rTF) and phospholipid in RC Low reagent were <3 pmol/L and <0.6 mmol/L, respectively. Relative fluorescence units were converted to thrombin generation (nmol/L) curves using Technoclone's evaluation software, which also calculated characteristic parameters such as peak thrombin concentration and endogenous thrombin potential (ETP).

2.6 | FVIII activity measurements

Factor VIII assay was measured by a chromogenic method using a commercial assay (Commute FVIII; Chromogenic by Diapharma). Calibration curves were produced using pooled normal plasma calibrated against the Whole Health Organization standard 91/666 for FVIII. FVIII concentrations were reported as a percentage, with a concentration of 100 IU/dL corresponding to 100%. All samples were run in duplicate. The intra-assay variation was 4%.

2.7 | Statistical analyses

Data obtained in this study are reported as mean ± standard error of the mean (SEM). Paired t test was used for comparing outcomes between two paired groups, and Pearson's or Spearman's correlation tests were used to derive correlation statistics. All statistical comparisons were two-tailed. The statistical significance threshold was set at 95% confidence level for all tests (P < 0.05).

3 | RESULTS

3.1 | Sensitivity to coagulation factor replacement therapy

Figure 2A shows the ClotChip read-out for blood samples collected from two haemophilic patients (one with inhibitors and one without) prior to the initiation of factor replacement therapy and 15 minutes after administration of appropriate coagulation therapy. As can be seen, the ClotChip recorded a substantially lower T_{peak} for post-therapy blood samples as compared to that for their pretherapy counterparts. Moreover, the observed decrease in the ClotChip T_{peak} parameter for the population was statistically significant in both cases: post-therapy samples compared with baseline (pretherapy) for children with haemophilia without inhibitors (Figure 2B; P = 0.0001, n = 9) and with inhibitors (Figure 2C; P = 0.028, n = 5).

Interestingly, the ClotChip read-out for baseline measurements of two tolerized patients with inhibitors exhibited T_{peak} values of 970 and 1010 seconds, which were within the range of T_{peak} values for patients without inhibitors (range: 740-1315 seconds; Figure 2D). However, the ClotChip T_{peak} for baseline measurements of three non-tolerized patients with inhibitors exhibited higher values of

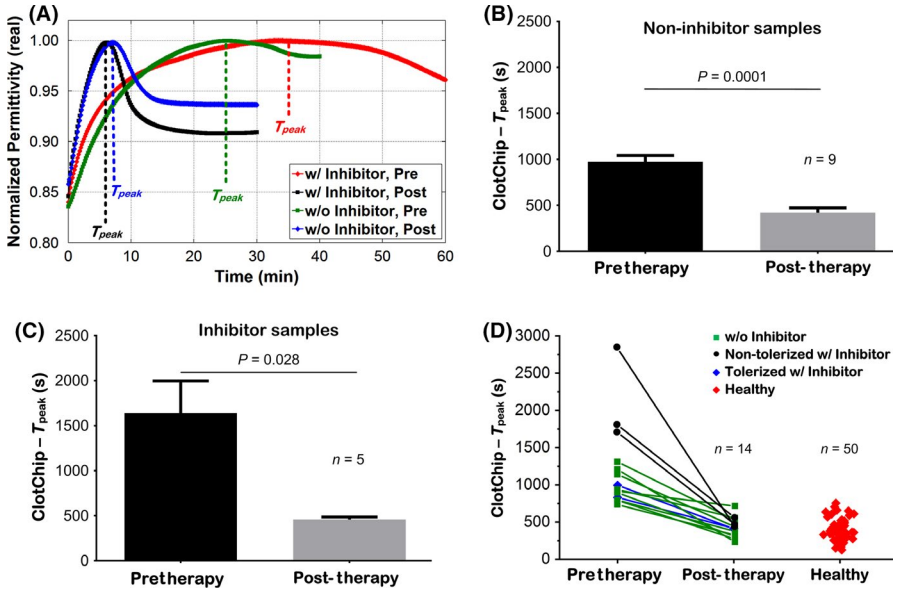

FIGURE 2 Effect of coagulation factor replacement therapy on ClotChip readout. A, Representative ClotChip curves from two children with haemophilia, with and without inhibitors at baseline (pre) and 15 min after initiation of factor replacement therapy (post). The time to reach a permittivity peak (T_{peak}), which is taken to be indicative of the coagulation time, decreased for post-therapy samples as compared to baseline. B, C, Paired *t* tests showed a significant decrease in ClotChip T_{peak} parameter for post-therapy samples as compared to baseline for patients without inhibitors (B, n = 9, $P = 0.0001$) and with inhibitors (C, n = 5, $P = 0.028$). D, Paired plot of ClotChip T_{peak} parameter for pre- and post-therapy samples (n = 14) and scatter plot of T_{peak} for healthy children (n = 50). All post-therapy samples fell within the reference range of healthy subjects. Error bars indicate duplicate measurements and are presented as mean ± standard error of the mean (SEM)

1710, 1810 and 2850 seconds (Figure 2D). Additionally, the ClotChip read-out for all post-therapy samples exhibited T_{peak} values that fell within the reference range for normal, healthy children (range: 125-720 seconds; Figure 2D). Taken together, these data suggest that the ClotChip T_{peak} parameter is sensitive to detection and correction of coagulopathy in children with haemophilia with and without inhibitors, as indicated by T_{peak} reaching reference-range values after coagulation factor replacement therapy.

3.2 | Correlation with parameters of ROTEM and TGA assays

We also carried out studies to investigate the correlative power of the ClotChip T_{peak} parameter with the ROTEM assay. Blood samples from nine haemophilia patients pre- and postadministration of factor replacement therapy were used for ClotChip measurements and were concurrently tested with non-activated ROTEM assay (Table 1). The ROTEM CT was used in this study as a clinically viable parameter to monitor patients with haemophilia. The ClotChip T_{peak} parameter exhibited a very strong positive correlation (Pearson's $r = 0.91$, $P < 0.0001$, n = 18) to the ROTEM CT parameter (Figure 3).

Similar experiments were also performed with TGA. Blood samples from three haemophilic patients pre- and postadministration of factor replacement therapy, as well as samples from five haemophilic patients for which a post-therapy sample was not available, were collected (Table 2). As seen in Figure 4, the ClotChip T_{peak} parameter exhibited a very strong negative correlation with ETP (Spearman's $r_s = -0.90$, $P = 0.0004$, n = 11) and peak thrombin (Spearman's $r_s = -0.92$, $P = 0.0002$, n = 11) parameters of TGA.

Hence, we have demonstrated a very strong correlation between the ClotChip T_{peak} parameter and clinically relevant parameters of two different assays for global assessment of haemostasis (ie, ROTEM and TGA). Taken together, these results demonstrate the ability of ClotChip to measure the global haemostatic potential of haemophilic patients.

3.3 | Sensitivity to plasma FVIII concentrations

Finally, we assessed the correlative power of the ClotChip T_{peak} parameter with the plasma FVIII concentrations generated from a chromogenic FVIII assay using the blood samples listed in Table 2. As seen in Figure 5, the ClotChip T_{peak} parameter exhibited a very strong negative correlation (Pearson's $r = -0.91$, $P < 0.0001$, n = 11) with FVIII concentrations (in $\log_{10}(\%)$), indicating once again the ClotChip potential for monitoring factor replacement therapy in haemophilia patients.

4 | DISCUSSION AND CONCLUSIONS

Our preliminary studies establish the feasibility of using the ClotChip for monitoring coagulation factor replacement therapy in severe haemophilia A patients with and without inhibitors, and indicate a promising correlative sensitivity of the ClotChip read-out to FVIII clotting activity and clinically relevant diagnostic parameters of ROTEM and TGA.

TABLE 1 Patient characteristics and results for ClotChip and ROTEM assays

Patient	Diagnosis	Therapy status	ClotChip T_{peak} (s)	ROTEM CT (s)
P1	HA w/ inhibitors	Pre	995	594
		Post	410	239
P2	HA w/ inhibitors	Pre	2850	2081
		Post	440	427
P3	HA	Pre	800	1327
		Post	380	313
P4	HA	Pre	1315	1199
		Post	530	265
P5	HA w/ inhibitors	Pre	1810	1499
		Post	560	354
P6	HA	Pre	920	530
		Post	720	441
P7	HA	Pre	945	560
		Post	560	425
P8	HA w/ inhibitors	Pre	830	368
		Post	415	250
P9	HA	Pre	740	-
		Post	330	-
P10	HA	Pre	1215	-
		Post	235	-
P11	HA w/ inhibitors	Pre	1710	1108
		Post	470	401

Note: Blood samples from patients with haemophilia A (HA) were collected both prior to the initiation of factor replacement therapy (pre) and 15 min after administration of an appropriate coagulation factor or bypassing agent (post). ClotChip measurements along with ROTEM were performed.

Patients with haemophilia and inhibitors have very little amount of FVIII activity at baseline, probably zero or between 0 and 1 IU/dL (0%-1%).[10] However, traditional FVIII assays (chromogenic and one-stage) are unable to measure FVIII levels below 1% reliably.[11,12] Our studies suggest that the ClotChip T_{peak} parameter was sensitive to the presence or absence of inhibitors, possibly measuring haemostatic potential that exists at FVIII levels between 0-1 IU/dL. We are conducting further studies to evaluate the ClotChip potential as a screening assay for detection of inhibitors in field settings.

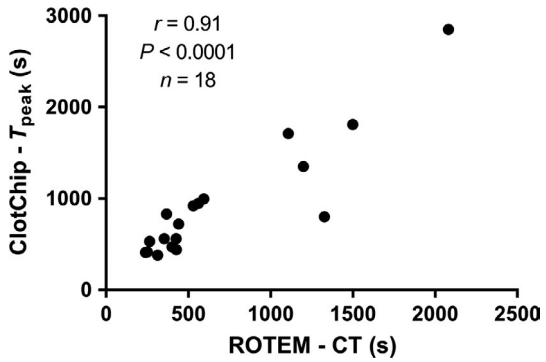

FIGURE 3 Comparison of ClotChip with rotational thromboelastometry (ROTEM). ClotChip T_{peak} parameter exhibited a very strong positive correlation with the clinically important clotting time (CT) parameter of ROTEM (Pearson's $r = 0.91$, $P < 0.0001$, n = 18)

Another global assay – clot waveform analysis – has been shown to be sensitive to very low levels (0-0.1 IU/dL) of FVIII and FIX as compared to TGA,[13] although the clinical significance of these very low factor levels in inhibitor patients is not currently understood. Although kaolin-activated TEG showed differences between inhibitor and non-inhibitor patients in one study,[14] other studies involving TEG and/or TGA have not. It is important to note that TEG, and not TGA, was able to differentiate between inhibitor and non-inhibitor patients. This can be explained by the fact that TEG is performed in whole blood as opposed to TGA, which is usually performed in PPP. Whole blood may add the contribution of minimal amounts of FVIII that remain in or on the platelets, even when no measurable FVIII activity is possible. Since ClotChip tests are also performed with whole blood, it is likely that ClotChip is able to measure global haemostasis.

The laboratory measurement of factor levels in patients with haemophilia is affected by various pre-analytical and analytical variables.[15] It has been questioned whether factor levels reflect the full haemostatic potential that exists at baseline and subsequent to factor replacement therapy in patients with haemophilia. Besides von Willebrand factor (vWF) levels, ABO blood group and

TABLE 2 Patient characteristics and results for ClotChip, TGA and FVIII assays

Patient	Diagnosis	Therapy status	ClotChip T_{peak} (s)	TGA ETP (nmol/L·min)	TGA Peak Thrombin (nmol/L)	FVIII Activity (%)
P11	HA w/ inhibitors	Pre	1710	0	0	1.10
		Post	470	-	-	-
P12	HA	Pre	1145	1263	69	11.2
		Post	440	2745	183	48.0
P13	HA	Pre	900	3134	156	1.0
		Post	310	3940	354	81.7
P14	HA	Pre	800	2581	90	4.3
		Post	275	3300	174	52.6
P15	HA	Pre	2535	0	0	0.48
P16	HA	Pre	1875	0	0	0.45
P17	HA	Pre	545	2239	117	12.9
P18	HA w/ inhibitors	Pre	2700	0	0	0.02

Note: Blood samples from patients with HA were collected, as described in Table 1, and tested with ClotChip, TGA and FVIII activity assays. For four patients, only a baseline (pre) blood sample was collected. FVIII activity was measured by the chromogenic method.

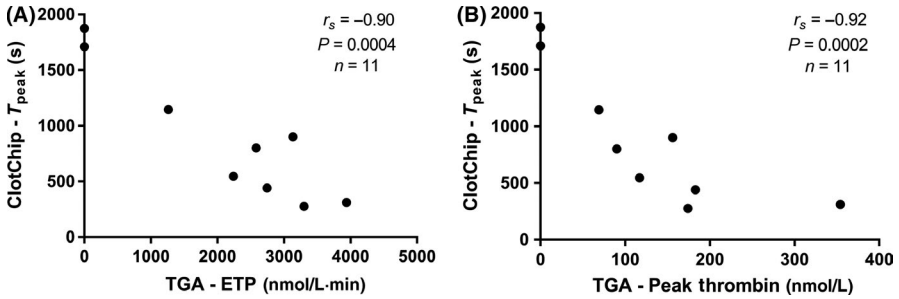

FIGURE 4 Comparison of ClotChip with thrombin generation assay (TGA). ClotChip T_{peak} parameter exhibited a very strong negative correlation with the clinically important A, endogenous thrombin potential (ETP) (Spearman's r_s = −0.90, P = 0.0004, n = 11) and B, peak thrombin (Spearman's r_s = −0.92, P = 0.0002, n = 11) parameters of TGA. Two data points at T_{peak} = 2535 s with ETP/peak thrombin = 0 and T_{peak} = 2700 s with ETP/peak thrombin = 0 are not shown

FV Leiden,[16-18] thrombin generation variability may affect a patient's bleeding phenotype. Hence, a reliable, point-of-care (POC), whole-blood assay with minimal pre-analytical variables can have tremendous potential for personalization of therapy in patients with haemophilia. The significant change in ClotChip T_{peak} readings before and after factor replacement therapy in our study demonstrates this potential. All of the post-therapy T_{peak} values came back down to levels that were within the reference range of control (normal) population (Figure 2D). We were able to demonstrate the ClotChip sensitivity with both FVIII in non-inhibitor patients (Figure 2B) and with bypassing agents in inhibitor patients (Figure 2C).

The inter-patient range of T_{peak} values at baseline in patients with inhibitors compared to those without inhibitors, as well as between patients without inhibitors, may also be a marker of the difference in clinical bleeding phenotype. In this study, we did not correlate the baseline T_{peak} values of patients with their known clinical bleeding phenotype. A prospective study to correlate the clinical bleeding phenotype to the ClotChip T_{peak} parameter is currently underway. The ability to differentiate between clinical bleeding phenotypes at presentation of haemophilia, or during therapy, can be a useful measure for timely initiation of prophylaxis, as well as to decide the

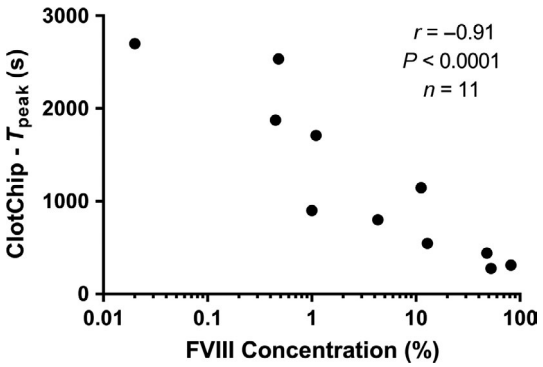

FIGURE 5 Comparison of ClotChip with FVIII assay. ClotChip T_{peak} parameter exhibited a very strong negative correlation with FVIII concentrations (in $\log_{10}$ (%)) obtained from a chromogenic FVIII assay (Pearson's r = −0.91, P < 0.0001, n = 11)

intensity of prophylaxis in terms of dose and frequency of factor replacement therapy.

Haemophilia patients with inhibitors present unique treatment and monitoring challenges.[19,20] There is limited ability, primarily using TEG and TGA, to monitor therapy with bypassing agents (recombinant FVIIa and activated prothrombin complex concentrates) in patients with inhibitors.[21-24] In our study, the change in ClotChip T_{peak} values pre- and post–bypassing agent therapy in inhibitor patients was statistically significant (Figure 2C; $P = 0.028$, n = 5).

We have also demonstrated excellent correlation of ClotChip T_{peak} to ETP (Spearman's $r_s = -0.90$, $P = 0.0004$, n = 11) and peak thrombin (Spearman's $r_s = -0.92$, $P = 0.0002$, n = 11) parameters of TGA, as well as to the CT parameter of ROTEM (Pearson's $r = 0.91$, $P < 0.0001$, n = 18). Furthermore, the T_{peak} values correlated very well to FVIII concentrations measured by the chromogenic assay (Pearson's $r = -0.91$, $P < 0.0001$, n = 11). In a study by Ljungkvist et al, the TGA-calibrated, automated thrombogram (TGA-CAT) and INNOVANCE ETP correlated well ($r = 0.734$ and $r = 0.701$, respectively) with FVIII clotting activity in haemophilia patients. However, the two methods of thrombin generation (TGA-CAT and INNOVANCE ETP) did not correlate with each other ($r = 0.546$).[25] The peak thrombin parameter of TGA has also been shown to correlate with clinical outcomes in haemophilia patients without inhibitors, although the TEG and FVIII activity measurements in the same study did not.[26] Although we did not document any clinical outcome measures in our study, an excellent correlation of our assay to all of the known monitoring methods in haemophilia is indeed promising.

Our study has several limitations, as we did not correlate the ClotChip T_{peak} parameter with the patients' clinical bleeding history, which would have demonstrated its ability to make meaningful changes in treatment decisions. A prospective study with the goal of establishing the ClotChip ability to tailor factor replacement therapy in patients with haemophilia without inhibitors is underway. We had a limited sample size and thus were unable to generalize our findings to the larger haemophilia community. Our study had only haemophilia A patients; hence, these findings are not directly applicable to haemophilia B patients. Moreover, we studied only severe haemophilia patients and not those with mild or moderate disease. Finally, our study had mostly children and a few young adults, so these findings will need confirmation in the older adult population.

In conclusion, our study shows that the ClotChip is a novel, POC, whole-blood assay with sensitivity to monitor coagulation factor replacement therapy in children with severe haemophilia A, with and without inhibitors. Using minuscule blood sample volumes (<10 μL), the ClotChip T_{peak} parameter exhibits a very strong correlation with salient parameters of TGA, ROTEM and chromogenic FVIII assay and, therefore, has potential for use in real-life settings to make decisions regarding coagulation factor replacement therapy in haemophilia A patients.

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DISCLOSURES

D. Maji, P. Mohseni and M. A. Suster are inventors of intellectual property that has been licensed by Case Western Reserve University to XaTek, Inc. M. A. Suster and P. Mohseni have received consulting fees and research funding from XaTek, Inc. S. P. Ahuja has received research funding from XaTek, Inc and fees from Bayer, Bioverativ and Shire.

AUTHOR CONTRIBUTIONS

D. Maji, L. Nayak and U. D. S. Sekhon performed experiments; L. Nayak, A. Sen Gupta, P. Mohseni, M. A. Suster and S. P. Ahuja conceptualized and planned experiments; S. P. Ahuja designed the clinical study; J. Martin and S. P. Ahuja obtained clinical samples; D. Maji, P. Mohseni and M. A. Suster prepared the figures; D. Maji, P. Mohseni, M. A. Suster and S. P. Ahuja wrote the manuscript.

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